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Temperature control of seed germination in *Fritillaria tubiformis* subsp. *moggridgei* (*Liliaceae*) a rare endemic of the South-west Alps

Valentina Carasso[1], Fiona R. Hay[2†], Robin J. Probert[2] and Marco Mucciarelli[1*]

[1]Department of Veterinary Morphophysiology, University of Torino, Via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy; [2]Seed Conservation Department, Royal Botanic Gardens Kew, Wakehurst Place, Ardingly, West Sussex RH17 6TN, UK

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Abstract

Fritillaria tubiformis subsp. *moggridgei* (*Liliaceae*) is a rare, endemic species that inhabits open mountains and alpine grasslands of the Ligurian Alps. At the time of seed dispersal, the underdeveloped embryos were 27% the length of the seed. Here we report the results of laboratory experiments carried out to determine the temperature preferences for embryo growth and radicle emergence. Embryo growth commenced immediately after sowing at 4°C. Once the embryo had grown the length of the seed, the radicle emerged. The time required for embryo growth and radicle emergence was longer when seeds were placed through a seasonal sequence of temperatures, commencing with late summer (10/20°C), compared with seeds immediately placed at a temperature to simulate winter conditions (4°C). Prematurely transferring seeds from winter to spring temperatures (5/10°C) also slowed the progress of germination. Radicle emergence did not occur at 10 or −5°C and less than 20% germination occurred in seeds placed at constant 0°C. Addition of gibberellic acid (GA_3) did not promote embryo growth of seeds placed at 20°C. Overall, the temperature preferences for embryo growth and subsequent radicle emergence are such that, *in situ*, seed germination may occur during the winter under snow cover or at the end of winter to coincide with snow melt and warming temperatures.

Keywords: alpine habitat, embryo growth, *Fritillaria tubiformis*, *Liliaceae*, seed germination

*Correspondence
Fax: +39 011 6709138
Email: marco.mucciarelli@unito.it

†Current address: TT Chang Genetic Resources Center, International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines.

Introduction

Fritillaria tubiformis Gren. & Godr. subsp. *moggridgei* (Boiss. & Reuter ex Planch.) Rix (*Liliaceae*) is a rare endemic species of the Ligurian and Maritime Alps, North West Italy (Gallino and Pallavicini, 2000; Conti *et al.*, 2005). It is a bulbous geophyte, with showy yellow flowers, growing on mountain and alpine grasslands between 800 and 2100 m above sea level (Pignatti, 1982). Many *Fritillaria* species originated from the Asiatic continent and the genus is widespread throughout Europe, North-west Africa, western North America and temperate Asia (Turrill, 1950). As a means of *ex situ* conservation, seeds of *F. tubiformis* subsp. *moggridgei* have been placed in storage at the Banca del Germoplasma, Parco Naturale Alte Valle Pesio e Tanaro, Chiusa Pesio, Cuneo, Italy. To be able to monitor the viability of the seeds in storage and to propagate them in case of future restoration of natural populations, as well as to understand the ecophysiology of this plant, the dormancy mechanism and germination preferences of the seeds need to be known.

Often temperate plants regulate the timing of seedling emergence at the soil surface to coincide with warming spring temperatures (Grime, 2001). In the Alps, prompt spring emergence is even more crucial in determining the chance of successful establishment, due to the relatively short period during which plants can benefit from snowmelt and compete with other plants. Timing of seedling emergence is mainly regulated by the dormancy mechanism and germination (radicle emergence) preferences of the seed (Vandelook *et al.*, 2009). However, after the seeds have germinated, there may also be a delay before seedling emergence due to either epicotyl dormancy (Baskin and Baskin, 1998) or a slow rate of growth (Karlsson *et al.*, 2005).

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Like other species in the *Liliaceae* (Kondo *et al.*, 2006), the embryos within the seeds of *F. tubiformis* are underdeveloped at the time of seed dispersal; the embryos need to grow to a critical length within the seed before germination can occur. Such a requirement for embryo growth prior to germination has been called morphological dormancy (Nikolaeva, 1977) and has been reported for species from seven of the nine genera of *Liliaceae* (Kondo *et al.*, 2006). Seeds with underdeveloped embryos may have an additional physiological block to germination; such seeds have been described as having morphophysiological dormancy (Baskin and Baskin, 1998). In this study, we wanted to determine the season when embryo growth occurred in *F. tubiformis* seeds and whether there was a physiological mechanism delaying radicle emergence. Specifically, we carried out laboratory experiments to determine: (1) the optimum temperature for embryo growth and the effect of gibberellic acid; (2) the effects of incubation at summer and autumn temperatures on subsequent radicle emergence; and (3) the effects of changes in winter duration on radicle emergence.

Materials and methods

Seed collection

Seeds of *Fritillaria tubiformis* subsp. *moggridgei* were collected from Pian del Lupo (2000 m above sea level), Alta Valle Pesio Natural Park (Piedmont, Italy) in August 2007 and 2008. Vegetation at Pian del Lupo is represented by an alpine meadow community (*Seslerietalia variae*) (Aeschimann *et al.*, 2004). Capsules with seeds were kept inside cotton bags during the transfer to the Valle Pesio seed bank where they were processed immediately. Seed quality and level of maturation was assessed under a stereoscope (×1.25) together with the number of empty seeds.

Embryo growth at seasonal versus low temperatures

Embryo growth within the intact seeds was measured for seeds placed through a seasonal cycle of temperatures [30 d at 10/20°C (summer) → 30 d at 5/10°C (autumn) → 150 d at 4°C (winter)] and for seeds placed directly at 4°C. For seeds collected in 2007, a sample of approximately 275 seeds was imbibed on filter paper moistened with distilled water at room temperature (20°C) in the dark for 24 h. The seeds were then sown, as subsamples of 25 seeds each, on 1% (w/v) water agar in 90-mm diameter Petri dishes. Images of the seeds in one dish selected at random were captured immediately after sowing, as described below, and the seeds then discarded. The remaining ten dishes were then divided between the two temperature regimes. A 12-h/12-h thermoperiod was used for the alternating temperature regimes and all experiments were carried out in the dark (dishes wrapped in aluminium foil). At 30-d intervals one dish was removed from each temperature regime for image capture, and then discarded.

Images of the intact seeds were acquired in Photoshop 7.0 using a CCD video camera (Nikon DS-Fi1) coupled to a stereomicroscope (Adobe Systems Inc., San Jose, California, USA). Because of the relatively transparent seed coat, it was possible to determine both the embryo and seed lengths using Image Pro-plus 6.2 (Media Cybernetics Inc., Silver Spring, Maryland, USA). Embryo lengths were not determined for seeds that had already germinated; embryo length is expressed as the mean for non-germinated seeds from an original sample of 25 seeds at each time point.

Radicle emergence at seasonal versus low temperatures

Soon after harvest in 2007, 32 samples of 25 seeds each were sown on 1% (w/v) water agar after 24-h on wetted filter paper, as above. Sixteen dishes were placed through a seasonal cycle of 30 d at 10/20°C (summer), 30 d at 5/10°C (autumn), and then placed at 4°C (winter) for either 30, 60, 90 or 120 d (four dishes for each period) before transfer to 5/10°C (spring). These temperature regimes were chosen to reflect average seasonal soil temperatures experienced *in situ*. A further 16 dishes were placed directly at 4°C (winter); four dishes selected at random were transferred to 5/10°C (spring) after 30, 60, 90 or 120 d. A 12-h/12-h thermoperiod was used for the alternating temperature regimes, and all dishes were wrapped in aluminium foil to exclude light. Seeds were checked weekly for radicle emergence in a dark room under green light. Seeds with protruded radicles were considered germinated and seeds that had not germinated by the end of the experiments were assessed with a cut test. A χ^2 test (maximum likelihood estimation) was carried out using GenStat 11 (VSN International Ltd., Hemel Hempstead, Herts, UK) to compare final germination proportions between treatments.

Radicle emergence at constant low temperatures

For seeds collected in 2008, four replicates of 25 seeds each were sown on 1% (w/v) water agar in 90-mm diameter Petri dishes and placed at each of −5, 0, 5 or 10°C. Petri dishes were wrapped with aluminium

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foil to exclude light. Seeds were checked for germination under green light in a dark room twice a week and seeds with a protruded radicle were removed. After 6 months, non-germinated seeds were moved to 5°C for a further 150 d to determine whether the seeds were still able to germinate.

Effect of GA_3 on embryo growth

Eight samples of 25 seeds each (collected 2008) were sown in 90-mm diameter Petri dishes on 1% (w/v) water agar containing 0, 100 or 250 mg l^{-1} gibberellic acid (GA_3; Sigma-Aldrich Inc., Dorset, UK). Dishes were sealed with Parafilm (Pechiney Plastic Packaging Company Inc., Chicago, Illinois, USA) to reduce water loss and wrapped in aluminium foil. For each concentration of GA_3, four dishes were placed at each of 4°C and 20°C. After 120 d, images of the intact seeds were captured, as before, for embryo length measurement.

Results

Embryo growth at seasonal versus low temperatures

At the time of seed dispersal, mean embryo length was 1.14 mm, 27% of the mean seed length. Embryo growth started as soon as seeds imbibed and proceeded at a relatively slow rate during the summer temperature regime (10/20°C; Fig. 1A); after 30 d the mean embryo length was 1.40 mm (33% of the length of the seed). Growth rate increased during the subsequent 30 d at the autumn temperature regime (5/10°C), and this rate was maintained for the first 30 d at the winter temperature (4°C). After 60 d at 4°C (120 d after sowing), the embryos in those seeds that had not yet germinated were close to reaching the full length of the seed.

The rate of embryo growth was faster in seeds placed directly at the winter temperature (4°C; Fig. 1B); at 60 d after sowing, mean embryo length was 3.0 mm, compared with 2.3 mm for seeds initially placed through summer and autumn regimes (Fig. 1A).

Radicle emergence at seasonal versus low temperatures

For seeds placed through the seasonal temperature sequence, germination (radicle emergence) was first observed 120 d after sowing (Fig. 1A). However, the rate of germination depended on when the seeds were transferred to the spring temperature (5/10°C). For seeds experiencing 60, 90 or 120 d at 4°C, prior to transfer to spring conditions (5/10°C), the rate and final percentage germination were broadly similar. For seeds given just 30 d at 4°C before transfer to 5/10°C, germination proceeded much more slowly and there was only 42% germination at 221 d after sowing.

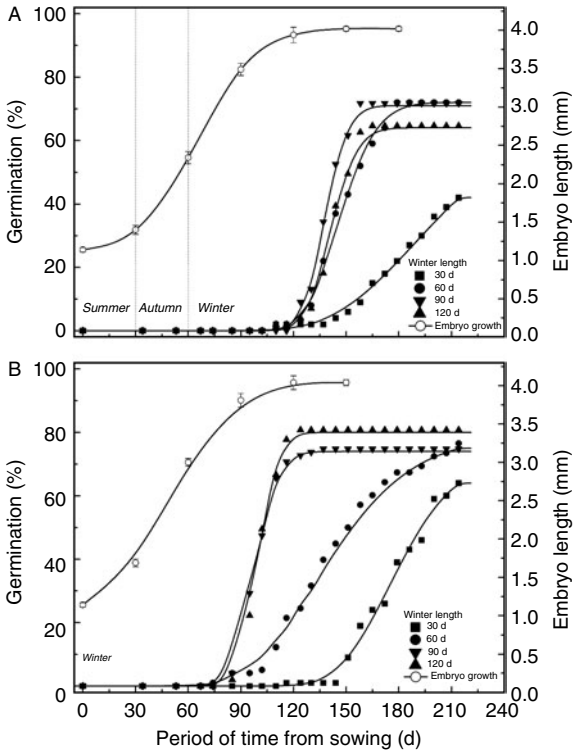

Figure 1. Embryo growth and germination progress curves of *Fritillaria tubiformis* subsp. *moggridgei* seeds. (A) Embryo growth (hollow symbols) and germination progress curves (filled symbols) for seeds placed through simulated summer (30 d at 10/20°C) and autumn (30 d at 10/5°C) 'seasons', then variable lengths of winter (30, 60, 90 or 120 d at 4°C), before transfer to spring (10/5°C) temperatures. Vertical lines indicate summer/autumn and autumn/winter temperature transitions. (B) Embryo growth (hollow symbols) and germination progress curves (filled symbols) for seeds placed directly into variable lengths of winter (30, 60, 90 or 120 d at 4°C), before transfer to spring (10/5°C).

When seeds were placed immediately at a winter temperature (no prior incubation at summer or autumn temperatures), germination was first observed at 75 d in seeds that remained at the winter temperature of 4°C for 90 or 120 d (Fig. 1B). In these treatments, the rate of germination was similar and there was not a significant difference in the final germination (reached 130 d after sowing) following transfer to 5/10°C ($P > 0.05$). When seeds were prematurely transferred to 5/10°C, after 60 or 30 d at 4°C, germination proceeded more slowly, with 75 and 64% germination, respectively, reached 210 d after sowing.

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Germination at constant low temperatures

Observations of embryo development when seeds collected in 2008 were held at a range of constant temperatures from −5 to 10°C revealed that all embryos failed to develop at −5°C (data not shown). However, when these seeds were transferred to 5°C after 181 d, the germination lag, progress and final germination were almost identical to those of seeds incubated directly at 5°C (Fig. 2). The embryos of a small proportion of seeds developed and germinated at 0°C. Following transfer to 5°C only three seeds germinated after a further 125 d and the remaining ungerminated seeds appeared to lose viability. Complete embryo development occurred in all seeds held at 5°C and 10°C but while all seeds germinated at 5°C after 150 d incubation, no seeds germinated at 10°C even after 181 d, when they were transferred to 5°C. Following transfer, there was no significant delay in germination, although the rate of germination, reflected by the slope of the germination progress curve, was clearly slower than in seeds incubated at 5°C throughout (Fig. 2).

Effect of GA_3 on embryo growth and germination

Addition of GA_3 (at either 100 or 250 mg l^{-1}) did not stimulate significant additional embryo growth in seeds placed at 20°C compared with seeds not given GA_3 and there was no germination of any seeds (with or without the addition of GA_3) at this temperature (Fig. 3). Similarly, for seeds placed at 4°C, the addition of GA_3 did not affect embryo growth (Fig. 3) or germination (~70%; data not shown) after 120 d at 4°C, compared with seeds not given GA_3.

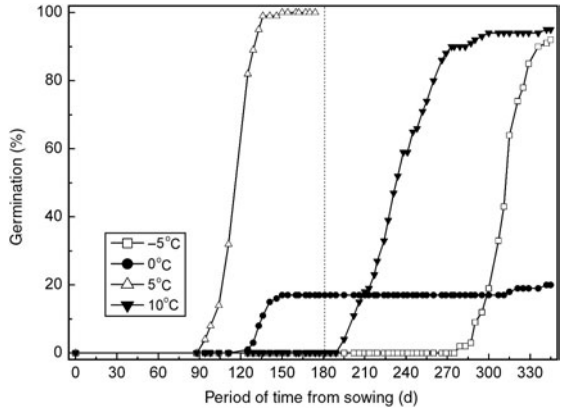

Figure 2. Germination progress curves for seeds of *Fritillaria tubiformis* (2008 harvest) when incubated at constant temperatures. The vertical dotted line at 181 d denotes when seeds, previously incubated at −5°C, 0°C and 10°C, were transferred to 5°C.

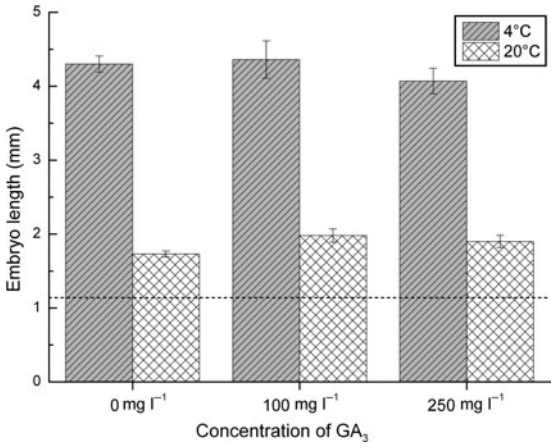

Figure 3. Mean (±SE) embryo length of *Fritillaria tubiformis* subsp. *moggridgei* seeds in response to different concentrations of gibberellic acid (GA_3). Seeds were incubated at 4 or 20°C for 140 d. The horizontal line shows the mean embryo length at the time of seed dispersal.

Discussion

Seeds of *Fritillaria tubiformis* subsp. *moggridgei* have an underdeveloped linear embryo (Martin, 1946). When they are dispersed towards the end of summer, the embryo must reach a critical length within the seed before germination can proceed. The critical embryo length varies between species that have underdeveloped embryos at the time of seed dispersal (Nikolaeva, 1999); in *F. tubiformis* subsp. *moggridgei*, the embryo has to grow the length of the seed before germination will occur. Embryo growth commenced immediately after sowing within seeds either moved through a seasonal sequence starting with a temperature regime that simulated late summer (10/20°C) or given a constant low temperature that simulated winter (4°C; Fig. 1). The rate of embryo growth was faster in seeds immediately placed at 4°C compared with seeds moved through the seasonal sequence. After 60 d at 4°C embryos were significantly larger (3.0 mm) compared with embryos in seeds that had experienced 30 d at summer followed by 30 d at autumn conditions (2.3 mm) (cf. Figs 1A and B).

The data presented raise an interesting question as to whether or not seeds of *F. tubiformis* exhibit physiological dormancy. Seeds with fully developed embryos, previously held at 10°C for 181 d, germinated without significant delay when transferred to 5°C and it could be argued that physiological dormancy had been removed during the period at 10°C. A number of previous studies on other species have indeed shown that while the optimum temperature for the release of physiological dormancy is around 5°C, dormancy can be broken at higher temperatures, albeit more slowly (for a review see Probert, 2000). The shallower slope of the germination progress curve after seeds had

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been incubated at 10°C is also consistent with the interpretation of less effective dormancy release during incubation at 10°C.

Non-dormant seeds, after the removal of physiological dormancy, would usually be expected to germinate over a wide range of temperatures (Baskin and Baskin, 1998; Finch-Savage and Leubner-Metzger, 2006) and therefore, if physiological dormancy had been broken during the 181 d period at 10°C, it is surprising that no seeds had germinated prior to transfer to 5°C. A simpler, and we believe more plausible, explanation is that seeds of *F. tubiformis* do not possess physiological dormancy. Rather, they simply possess a very narrow, low, optimum range of temperatures for germination. Although 10°C is suitable for embryo growth, it is too high to support germination, whereas temperatures around 4–5°C appear to be optimal for both processes. The fact that complete embryo development was possible at 10°C and that germination occurred without significant delay when seeds were subsequently transferred to 5°C (Fig. 2) indicates that the seeds did not require a period of cold stratification at 5°C in order to overcome a physiological block to germination. It can be argued that the only reason why germination is delayed in *F. tubiformis* is simply because embryos need to grow first and as soon as this process is complete, germination will occur as long at the temperature is favourable.

The slower germination of seeds following incubation at 10°C (indicated by the shallower progress curve) compared with seeds held at constant 5°C (Fig. 2) could be explained by loss in vigour. Although detailed observations were not made, based on the rate of embryo growth measured during incubation for 30 d at autumn temperatures (5/10°C) (Fig. 1), it is likely that embryo elongation at 10°C would have been complete in all seeds considerably sooner than the 181 d incubation period. It is therefore likely that some deterioration and loss of vigour would have occurred prior to transfer to 5°C.

The apparent loss of viability in seeds held for a prolonged period at 0°C but not at other temperatures is difficult to explain and warrants further investigation. Sub-zero soil temperatures are likely to be experienced only if snow cover is thin or absent during the winter. Under thick snow, soil temperatures are likely to be slightly above freezing (Milbau *et al.*, 2009) and this situation is more typical of the sites inhabited by *F. tubiformis*. Although we did not test germination at temperatures between 0°C and 4°C, it seems likely that seeds would have been able to germinate in this range and we predict that germination in the wild probably can occur during winter under snow cover.

In contrast with other species in which, once the embryos have reached their critical length, there is a mechanism that delays germination, *F. tubiformis* subsp. *moggridgei* embryos continued to grow and the radicle emerged as long as the temperature was favourable. In *Aegopodium podagraria*, although the embryo only grew at 5°C, radicle emergence did not occur immediately after the completion of embryo growth; an additional cold stratification period was necessary for germination to proceed (Vandelook *et al.*, 2009). The commencement of embryo growth immediately after dispersal has also been reported in, for example, *Anemone nemorosa* seeds placed under a temperature regime that simulated conditions experienced *in situ* following seed dispersal (Mondoni *et al.*, 2008). In *Caltha leptosepala*, a species of alpine wet meadows, embryo growth takes place beneath snowbanks and germination occurs at 2.5°C (Forbis and Diggle, 2001). Clearly, temperature preferences will depend on the timing of seed dispersal and the conditions normally experienced following dispersal and, as for some other alpine species (Forbis and Diggle, 2001; Liebst and Schneller, 2008), the restriction of germination to a narrow range of low temperatures allows *F. tubiformis* subsp. *moggridgei* plantlets to be safely established by the end of the winter.

Examination of the data presented in Figs 1A and B reveals that as long as embryos were more or less fully developed at the time of transfer to the spring temperature of 5/10°C, germination occurred quickly and to a high percentage. When seeds were transferred to 5/10°C before embryos were fully developed, germination was slower. This reduction in the speed of germination is unlikely to be explained by reduced embryo growth, which clearly can occur even at constant 10°C. A more likely explanation is that since germination cannot be supported at 10°C (Fig. 2) seeds need longer to accumulate time at the optimum temperature for germination (5°C). Indeed, there is a good relationship between total time at 4 or 5°C and percentage germination, regardless of the regimes experienced.

Conclusions

Contrary to previous reports, in this paper we have presented evidence suggesting that seeds of *F. tubiformis* subsp. *moggridgei* may not possess physiological dormancy, and the same might be true for other species of *Fritillaria*. A period of embryo growth inside the seeds immediately after dispersal, while ambient temperatures are low, ensures that radicle emergence in this species does not occur until conditions, either during or at the end of the winter, are suitable.

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